Data are expressed as means ± S.D. $P<0.05$ was considered statistically significant.

Results

BCTS Stimulates Breast Cancer Cell Growth in Both Estrogen-Dependent and -Independent Manners

In the tumor microenvironment, many growth factors, cytokines and chemokines directly and indirectly control growth. To study their comprehensive influence on breast cancer aggressiveness, we first analyzed effects of BCTS on MCF-7-E10 cell growth (Fig. 1), which allowed us to examine the total effect of breast cancer-derived factors secreted from tumor and stromal cells, as they exist in vivo, on growth and estrogen-related signals of breast cancer cells. BCTS dose-dependently stimulated MCF-7-E10 cell growth (Fig. 1a). Although activities varied among specimens, more than 60 % showed higher growth-stimulating activity than with estrogen (Fig. 1b).

To examine the specificity of target cells, we studied the effect of BCTS on growth of other tumor cell lines, including a breast cancer cell line, T47D, a lung adenocarcinoma cell line, PC9, and a cervical cancer cell line, HeLa (Fig. 2a). The growth of T47D, another ER + human breast cancer cell line, was stimulated by BCTS while growth of PC9 was not increased. HeLa cell growth was rather inhibited by BCTS. The growth of MDA-MB-231 cells, an ER– human breast cancer cell line, was also stimulated by the extracts (data not shown). These results suggest that BCTS specifically stimulated breast cancer cell growth regardless of ER expression.

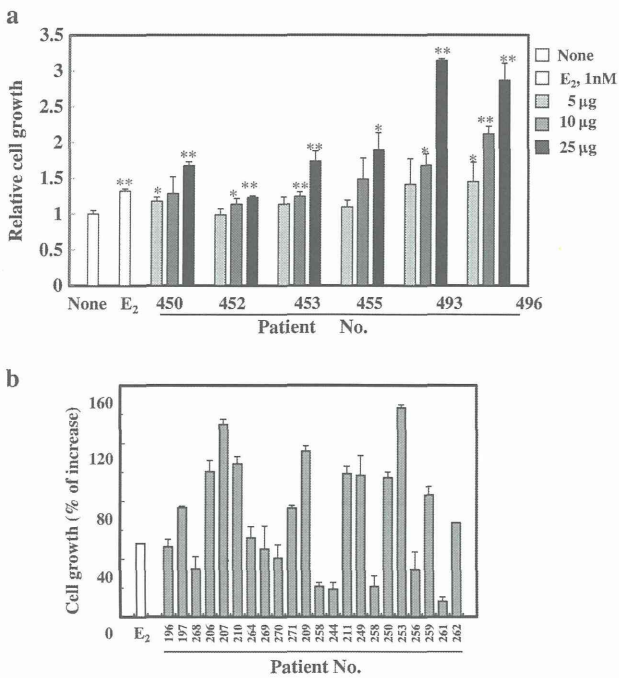

Fig. 1 BCTS effectively stimulated growth of MCF-7-E10 cells. After 3 days of culture in estrogen-deprived medium, MCF-7-E10 cells were cultured with breast cancer tissue supernatant at the indicated protein concentrations (**a**) or at 25 μg (**b**) in total 150 μl medium per well in 96-well plate for 4 days. The viable cells were examined using a Cell Counting Kit-8 assay. Values relative to control are shown. Data are presented as mean ± SD of triplicate determinations. *, $P<0.05$; **, $P<0.01$

Next, to see whether growth-stimulating activity in the tissue supernatant affected only the tumoral region, we analyzed extracts of tumoral regions and non-tumoral regions 2 cm distal to the tumor. The tumoral regions had more growth-stimulating activity than the non-tumoral regions (Fig. 2b), suggesting that the tumoral regions have an abundance of growth-stimulating activities for breast cancer cells.

To see if ER activation was required for BCTS-induced growth stimulation, we analyzed GFP expression in MCF-7-E10 cells, and found growth stimulation was not necessarily accompanied by ER activation (Fig. 3a). We next examined effects of anti-estrogen agents such as tamoxifen and fulvestrant on BCTS-induced growth stimulation, and found that high growth-stimulating activities were resistant to fulvestrant (Fig. 3b) and tamoxifen (Fig. 3c). These results indicate that, in addition to an ER-dependent pathway, BCTS stimulates breast cancer growth via an ER-independent pathway.

Growth-Stimulating Activity Correlated with Clinicopathological Characteristics

We analyzed the relationships between ER-independent growth-stimulating activity detected in BCTS and clinicopathologic characteristics of the specimens' donors (Fig. 4). Although BCTS growth-stimulating activity did not correlate with expression of ERα or PgR, stage, menopausal status, grade or nodal status (data not shown), specimens from tumors larger than 10 mm showed higher growth-stimulating activity than those smaller than 10 mm (Fig. 4a). Breast cancers are categorized into four intrinsic subtypes according to gene-expression profile: luminal A (ER + and/or PgR+, HER2–), luminal B (ER + and/or PgR+, HER2+), HER2 (ER–, PgR–, HER2+) and basal-type (ER–, PgR–, HER2–) [24, 25]. BCST derived from HER2 subtype showed slightly or significantly higher growth-stimulating activity than that from luminal B or basal types, respectively (Fig. 4b), suggesting that the tumor extracts of HER2 subtype have an abundance of growth factors stimulating their own receptors, including those of the ERBB family.

We next analyzed relationships between HER2 expression and growth-stimulating activity in ER– breast cancers, and found that the cases with high growth-stimulating activity differed significantly from those with low activity in terms

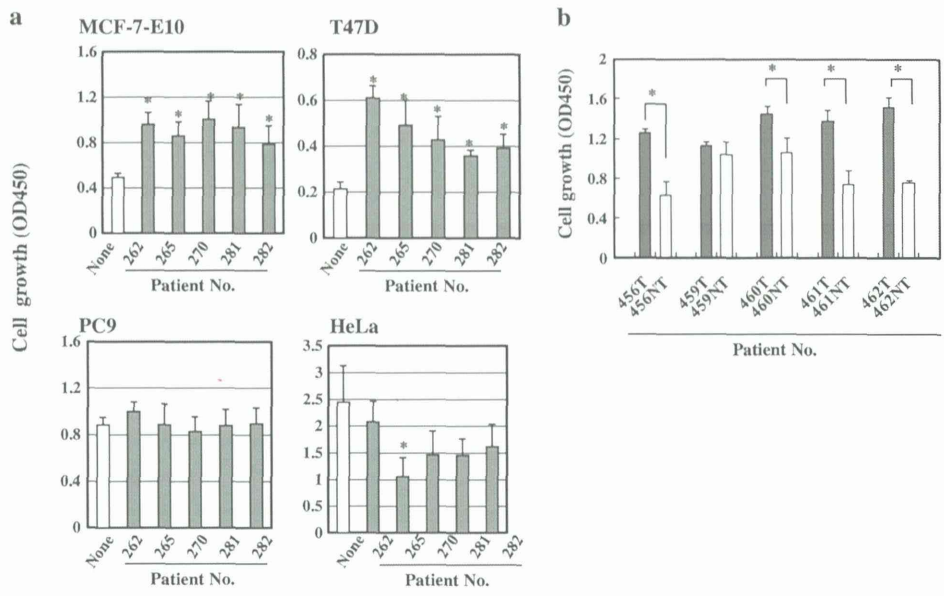

Fig. 2 Specificity of cell growth-stimulating activity detected in BCTS. **a** BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate effectively stimulated growth of breast cancer cell lines, MCF-7-E10 and T47D which were precultured in estrogen-deprived medium for 3 days. The viable cells were examined using a Cell Counting Kit-8 assay. **b** Specificity for BCTS-derived region. T, tissue supernatant derived from tumor region; NT, tissue supernatant derived from the region 2 cm distal to the tumor region. Data are presented as mean ± SD of triplicate experiments. *, $P<0.05$

of HER2 expression; more specimens with high HER2 scores (score 3) were seen among cases with high activity (Fig. 4c). This difference could not be observed for ER + breast cancers.

Breast cancers have histological types that reflect biological characteristics. Invasive ductal carcinoma can be classified into three subtypes—papillotubular, solid-tubular and

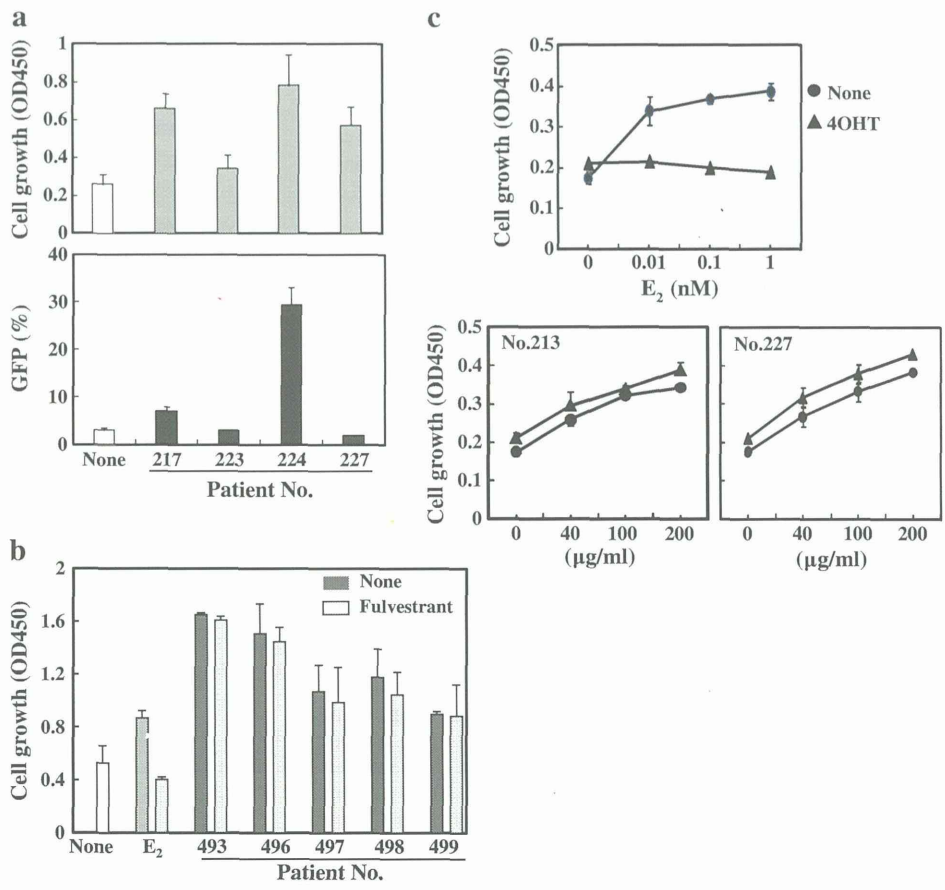

Fig. 3 BCTS stimulated growth of MCF7-E10 cells in an estrogen-independent manner. **a** MCF7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate for 4 days. Cell growth was examined using a Cell Counting Kit-8 assay and ER activities are shown as the percentage of MCF-7-E10 cells expressing GFP. **b**, **c** MCF-7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate or the indicated concentrations in the presence or absence of anti-estrogen agents, fulvestrant (1 μM) or 4-hydroxy tamoxifen (4OHT, 1 μM), for 4 days. 17β-Estradiol (E_2) was also tested at 1 nM or the indicated concentrations. Cell growth was examined using a Cell Counting Kit-8 assay. Data are presented as mean ± SD of triplicate experiments

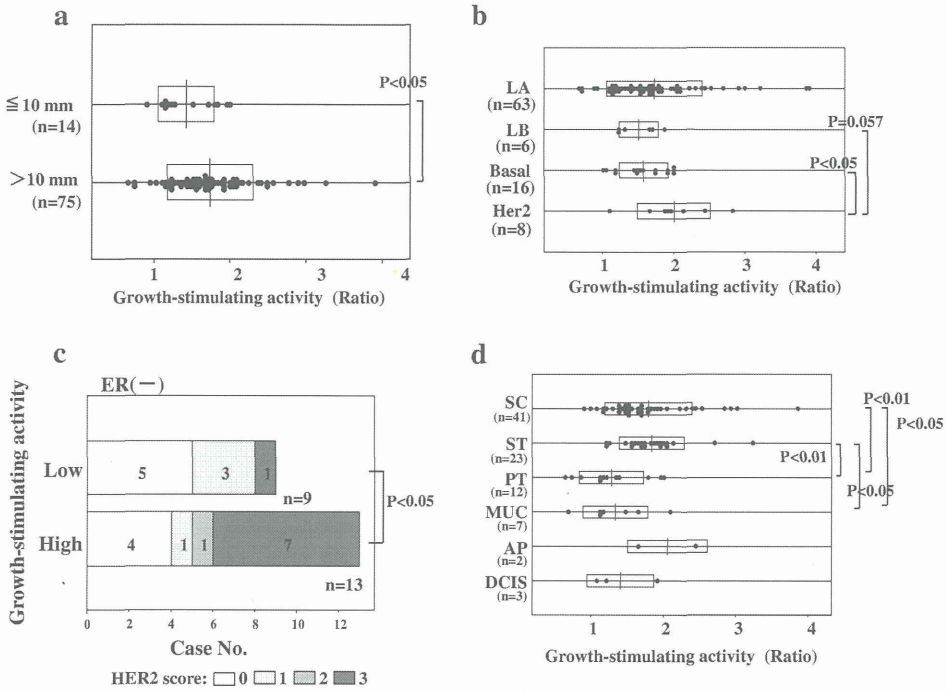

Fig. 4 Correlations between growth-stimulating activity and clinicopathological characteristics, intrinsic subtypes and histological subtypes. MCF7-E10 cells were cultured with BCTS at 25 μg protein concentration in 150 μl medium per well in 96-well plate for 4 days. Cell growth was examined as described in Materials and Methods for triplicate experiments, and the growth-stimulating activities are shown as the ratios calculated relative to the control. Data are presented as mean ± SD of triplicate experiments. High growth-stimulating activity in specimens was associated with tumor size (**a**), intrinsic subtype (**b**), HER2 expression in ER-negative breast cancer (**c**) or histological classifications (**d**). Differences between groups were determined by two-sample *t*-test. $P<0.05$ was considered statistically significant

scirrhous carcinoma—which are related to prognosis. We previously reported their relative overall survival rates as papillotubular carcinoma > solid-tubular carcinoma > scirrhous carcinoma [26]. The more aggressive scirrhous carcinoma and solid-tubular carcinoma show higher growth-stimulating activity than do papillotubular carcinoma and mucinous types (Fig. 4d), suggesting that growth-stimulating activity is related to aggressiveness in breast cancer.

Growth Factors in BCTS Promote MCF-7-E10 Cell Growth

Growth-stimulating activity was heat labile and detectable in the fraction with an MW greater than 5 kDa (data not shown), suggesting that it could be derived from proteinous factors. Among various factors in the tumor microenvironment, HGF derived from stromal fibroblasts has been reported to stimulate growth of mouse mammary tumor cells in primary culture [27]; EGF and IGF-1 are known to activate ER via phosphorylation [18, 19]. To analyze the participation of these growth factors in tumor growth-stimulating activities found in BCTS, we first examined the effect of anti-HGF antibody on them. As shown in Fig. 5a, anti-HGF antibody, but not control IgG, effectively inhibited extract-stimulated growth of MCF-7-E10 cells. MCF-7 cells reportedly express c-Met, a receptor for HGF.

We next analyzed the roles of EGF and IGF-1, using the inhibitors specific for their receptors. IGF-R inhibitor dose-dependently inhibited the growth of MCF-7-E10 cells while EGF-R inhibitor, in contrast, stimulated their growth (Fig. 5b, c).

Finally, we analyzed growth factors present in BCTS using the enzyme immunoassay. HGF was detected in more than 70 % of the tested samples, whereas EGF was detected only in 3 out of 25 samples (Fig. 5d). Although the analysis using IGF-1R inhibitor suggested involvement of IGF-1 in the growth-stimulating effect of BCTS as described above, IGF-1 could not be detected in the enzyme immunoassay. This might be because of the immunoassay's sensitivity, or because other ligands for IGF-1R (such as IGF-II, insulin or unknown factors) might have been present in the tumor extracts. These results suggest that signal pathways via HGF or IGF-1R play a significant role in promoting the growth of breast cancer cells.

Discussion

The tumor microenvironment is apparently associated with important aspects of epithelial solid tumor progression, including tumor growth, angiogenesis and metastasis. In the tumor microenvironment, growth factors such as EGF, IGF-1, transforming growth factor-α, transforming growth factor β

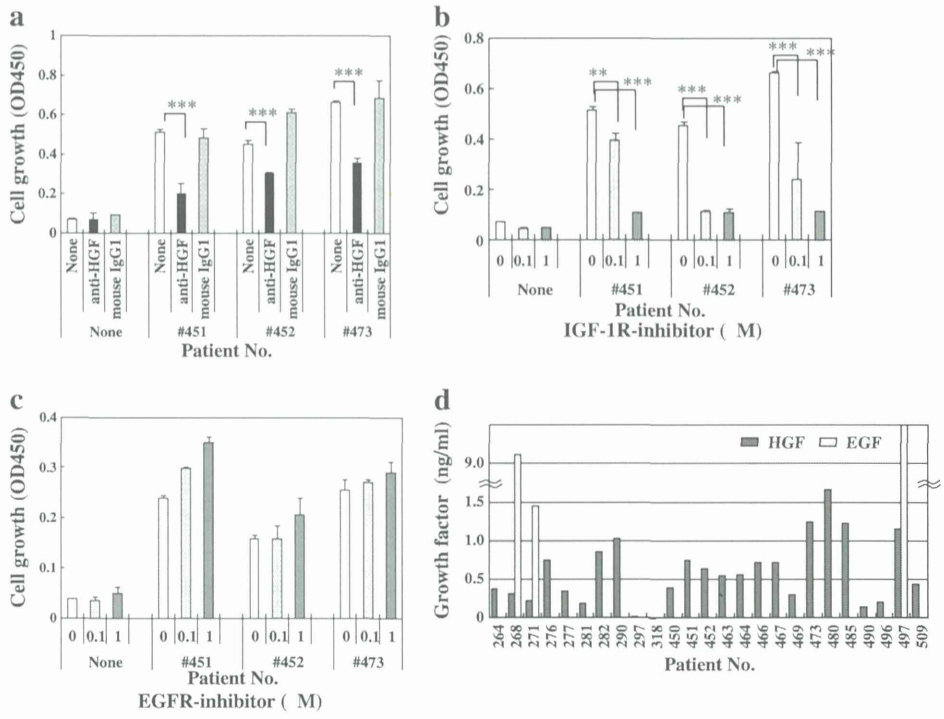

Fig. 5 Detection of growth factors involved in growth-promoting activity for MCF-7-E10 cells in BCTS. MCF-7-E10 cells were incubated with BCTS in the presence of anti-HGF antibody (**a**), AG1024, IGF-1R inhibitor (**b**), or AG1478, EGFR inhibitor (**c**), at the indicated concentrations. For anti-HGF antibody treatment, BCTS was pre-incubated with anti-HGF antibody for 30 min at room temperature and was then used for assay. Mouse IgG1 antibody was used as an isotype control. **d** The concentrations of HGF and EGF detected in BCTS were analyzed by immunoassay using Quantikine (R&D Systems, MN, USA). **, $P<0.01$, ***, $P<0.001$

and stromal-derived factor-I reportedly affect breast cancer growth, directly or indirectly [1, 3, 4]; however, the combined effects of these factors and their signal interactions in vivo are unclear. In this study, using the supernatant of breast cancer tissues, we analyzed the comprehensive effects of breast cancer-derived factors and found that BCTS effectively and specifically stimulated breast cancer cell growth. In addition to estrogen, which is locally produced in the microenvironment in breast cancers of postmenopausal patients [6, 8], our results suggest that the tumor extracts also stimulated breast cancer cell growth in an estrogen-independent manner, as anti-estrogen agents such as tamoxifen and fulvestrant did not inhibit the effect of BCTS. Furthermore, clinicopathological data and BCTS-associated growth-stimulation correlated with tumor size and HER2 expression, indicating the physiological significance of growth-stimulating activity in BCTS. Thus, BCTS offers an appropriate means to analyze the combined effect of the breast cancer-derived factors on tumor cell behavior.

Although many growth factors might be present in BCTS, we found HGF and IGF-1R-related signals to affect the growth-stimulating activity of BCTS, because it was suppressed by anti-HGF antibody and IGF-1R inhibitor. HGF was detected in tissue extracts of more than 70 % of breast cancer specimens whereas EGF was detected in only 12 % (Fig. 5d). The growth-stimulating activities did not always correlate with HGF concentrations (data not shown), but this is expected, as growth-stimulating activities in the supernatant are derived from the signal cross-talks of several factors. HGF, which acts through its receptor MET, is a multifunctional cytokine that induces cell survival, growth, differentiation and motility in most solid human cancers including colorectal, renal and breast cancers [28]. In normal epithelial cells, HGF, in combination with other growth factors, promotes mammary ductal morphogenesis [29]. Overexpression of both HGF and MET have been frequently reported in breast cancers, and are associated with poor prognosis [30]. HGF reportedly stimulates breast cancer growth in a paracrine fashion, in that HGF is produced primarily by stromal fibroblasts and acts on epithelial cells through its receptor MET [27, 31]. Stromal fibroblasts from breast cancer tissue produce large amounts of HGF compared with normal fibroblasts [30]. A c-Met-targeted therapy, ARQ197—which selectively targets c-Met tyrosine kinase—is currently in a phase II clinical trial [32]; SGX523—a novel ATP-competitive inhibitor, that is exquisitely selective for inhibition of MET-mediated signaling—is also being developed [33].

We found that IGF-1R signaling mediated the growth-stimulating activity of BCTS, because IGF-1R-specific inhibitor decreased the growth-stimulating effect of BCTS. IGF-1R-related signals are widely shown to induce cell proliferation and survival in breast cancer [34–36]; IGF-R1 activation protects breast cancer cells from apoptosis induced by various anticancer drugs [37]. While BCTS stimulated growth of MCF-7-E10 cells in an estrogen-independent manner, functional interactions between estrogen and IGF-1R signaling

pathways, including Ras/MAPK and PI3K/Akt have been reported [38] Estrogen also up-regulates IGF-1R expression in breast cancer [36]. However, we could not detect IGF-1 and stromal cell-derived growth factor-1alpha in BCTS (data not shown), possibly because of the limit of sensitivity by the immunoassay used in our study; or that other ligands may be present in the breast cancer microenvironment that activate IGF-1R—including IGF-II, insulin and unknown factors [39]. Indeed, overexpression of IGF-1R in MCF-7 cells has been shown to induce IGF-1R tyrosine kinase activation in the absence of exogenous IGF-1 [40].

These results suggest that signaling pathways via HGF/c-Met or IGF-1R significantly affect breast cancer cell growth. However, growth-stimulating activity found in BCTS might be derived from orchestrated signal crosstalks of several factors, because recombinant growth factors, including HGF and IGF-1, could not induce MCF-7-E10 cell growth when used alone. Further investigations of these activities and the identification of the cellular sources of the growth factors are needed to identify the mechanisms of the growth-stimulating effect of breast cancer tissue supernatant, which may help design more effective targeted therapies for breast cancer.

Conclusions

The breast cancer microenvironment provides estrogen and growth factors that affect tumor behavior, but the comprehensive effects of these factors, including signal crosstalk, on progression of breast cancer remain unclear. Using an estrogen-signal reporter cell line, MCF-7-E10, stably transfected with the ERE-GFP gene, we analyzed the effect of factors present in breast cancer tissues to reflect the in vivo status of individual cases. We found that they stimulated growth of MCF-7-E10 cells in an estrogen-independent manner, and that growth-promoting activity is related to aggressiveness in breast cancer. Moreover, signal pathways via HGF and IGF-1 receptor were involved in these activities. Our study strongly suggests that the evaluation of comprehensive tumor-promoting activity for individual breast cancers is important in determining appropriate therapy.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Advanced Research for Medical Products Mining Programme of the National Institute of Biomedical Innovation (NIBIO).

Competing interest The authors declare that they have no competing interest.

Authors' contributions YY and SH were involved in experimental design, performed all experiments, and drafted the manuscript. YS assisted in experiments and performed statistical analysis of the data. HT participated in acquisition and interpretation of the clinical data of patients. MK participated in experimental design and histological evaluation. All authors contributed to the analysis of data and approved the final manuscript.

References

1. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A et al (2004) Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6:17–32
2. Mueller MM, Fusenig NE (2004) Friends or foes: bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4:839–849
3. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348
4. Yamaguchi Y (2007) Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 14:175–181
5. Yamaguchi Y, Hayashi S (2009) Estrogen-related cancer microenvironment of breast carcinoma. Endocr J 56:1–7
6. O'Neill JS, Miller WR (1987) Aromatase activity in breast adipose tissue from women with benign and malignant breast diseases. Br J Cancer 56:601–604
7. Santen RJ, Santner SJ, Pauley RJ, Tait L, Kaseta J, Demers LM, Hamilton C, Yue W, Wang JP (1997) Estrogen production via the aromatase enzyme in breast carcinoma: which cell type is responsible? J Steroid Biochem Mol Biol 61:267–271
8. Simpson ER, Davis SR (2001) Minireview: aromatase and the regulation of estrogen biosynthesis–some new perspectives. Endocrinology 142:4589–4594
9. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER (1997) Transcriptional regulation of CYP19 gene (aromatase) expression in adipose stromal cells in primary culture. J Steroid Biochem Mol Biol 61:203–210
10. Smith IE, Dowsett M (2003) Aromatase inhibitors in breast cancer. N Eng J Med 348:2431–2442
11. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M et al (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 28:509–518
12. Hayashi S, Yamaguchi Y (2005) Estrogen signaling and prediction of endocrine therapy. Cancer Chemother Pharmacol 56:27–31
13. Hayashi S, Niwa T, Yamaguchi Y (2009) Estrogen signaling pathway and its imaging in human breast cancer. Cancer Sci 100:1773–1778
14. Hayashi S (2004) Prediction of hormone sensitivity by DNA microarray. Biomed Pharmacother 58:1–9
15. Inoue A, Yoshida N, Omoto Y, Oguchi S, Yamori T, Kiyama R, Hayashi S (2002) Development of cDNA microarray for expression profiling of estrogen-responsive genes. J Mol Endocrinol 29:175–192
16. Inoue A, Omoto Y, Yamaguchi Y, Kiyama R, Hayashi S (2004) Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells. J Mol Endocrinol 32:649–666
17. Yoshida N, Omoto Y, Inoue A, Eguchi H, Kobayashi Y, Kurosumi M, Saji S, Suemasu K, Okazaki T, Nakachi K et al (2004) Prediction of prognosis of estrogen receptor-

positive breast cancer with combination of selected estrogen-regulated genes. Cancer Sci 95:496–502
18. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H et al (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491–1494
19. Osborne CK, Shou J, Massarweh S, Schiff R (2005) Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. Clin Cancer Res 11:865s–70s
20. Yamaguchi Y, Takei H, Suemasu K, Kobayashi Y, Kurosumi M, Harada N, Hayashi S (2005) Tumor-stromal interaction through the estrogen-signaling pathway in human breast cancer. Cancer Res 65: 4653–4662
21. Allred DC, Harvey JM, Berardo M, Clark GM (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 11:155–168
22. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A et al (2007) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25: 118–145
23. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19: 403–410
24. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS et al (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98:10869–10874
25. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S et al (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100:8418–8423
26. Kurosumi M, Tabei T, Inoue K, Takei H, Ninomiya J, Naganuma R, Suemasu K, Higashi Y, Tsuchiya E (2003) Prognostic significance of scoring system based on histological heterogeneity of invasive ductal carcinoma for node-negative breast cancer patients. Oncol Rep 10: 833–837
27. Sasaki M, Enami J (1999) Mammary fibroblast-derived hepatocyte growth factor and mammogenic hormones stimulate the growth of mouse mammary epithelial cells in primary culture. Endocrine J 44: 359–366
28. Christensen JG, Burrows J, Salgia R (2005) c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett 225:1–26
29. Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C, Birchmeier W (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol 131:215–226
30. Yamashita J, Ogawa M, Yamashita S, Nomura K, Kuramoto M, Saishoji T, Shin S (1994) Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res 54:1630–1633
31. Tyan SW, Kuo WH, Huang CK, Pan CC, Shew JY, Chang KJ, Lee EY, Lee WH (2011) Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PLos ONE 6:e15313
32. Munshi N, Sébastien J, Li Y, Chang-Rung C, France DS, Ashwell MA, Hill J, Moussa MM, Leggett DS, Li CJ (2010) ARQ197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase and antitumor activity. Mol Cancer Ther 9:1544–1553
33. Buchanan SG, Hendle J, Lee PS, Smith CR, Bounaud PY, Jessen KA, Tang CM, Huser NH, Felce JD, Froning KJ et al (2009) SGX523 is an exquisitely selective, ATP-competitive inhibitor of the MET receptor tyrosine kinase with antitumor activity in vivo. Mol Cancer Ther 8:3181–3190
34. Resnik JL, Reichart DB, Huey K, Webster NJG, Seely BL (1998) Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. Cancer Res 58:1159–1164
35. Law JH, Habibi G, Hu K, Masoudi H, Wang MY, Stratford AL, Park E, Gee JM, Finlay P, Jones HE et al (2008) Phosphorylated insulin-like growth factor-i/insulin receptor is present in all breast cancer subtypes and is related to poor survival. Cancer Res 68:10238–10246
36. Maor S, Mayer D, Yarden RI, Lee AV, Sarfstein R, Werner H, Papa MZ (2006) Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1. J Endocrinol 191: 605–612
37. Dunn SE, Hardman RA, Kari FW, Barrett JC (1997) Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. Cancer Res 57:2687–2693
38. Stoica GE, Franke TF, Moroni M, Moroni M, Mueller S, Morgan E, Iann MC, Winder AD, Reiter R, Wellstein A, Martin MB, Stoica A (2003) Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. Oncogene 22:7998–8011
39. Macaulay VM (1992) Insulin-like growth factors and cancer. Br J Cancer 65:311–320
40. Surmacz E, Burgaud JL (1995) Overexpression of insulin receptor substrate 1 (IRS-1) in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation. Clin Cancer Res 1:1429–1436

Journal of Steroid Biochemistry & Molecular Biology 144 (2014) 513–522

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Increased androgen receptor activity and cell proliferation in aromatase inhibitor-resistant breast carcinoma

Rika Fujii [a,b], Toru Hanamura [a], Takashi Suzuki [c], Tatsuyuki Gohno [a], Yukiko Shibahara [d], Toshifumi Niwa [a], Yuri Yamaguchi [f], Koji Ohnuki [g], Yoichiro Kakugawa [h], Hisashi Hirakawa [i], Takanori Ishida [b], Hironobu Sasano [d], Noriaki Ohuchi [b], Shin-ichi Hayashi [a,e,*]

[a] Department of Molecular and Functional Dynamics, Tohoku University Graduate School of Medicine, Sendai, Japan
[b] Department of Surgical Oncology, Tohoku University Graduate School of Medicine, Sendai, Japan
[c] Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, Sendai, Japan
[d] Department of Anatomic Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan
[e] Department of Center for Regulatory Epigenome and Diseases, Tohoku University Graduate School of Medicine, Sendai, Japan
[f] Research Institute for Clinical Oncology, Saitama Cancer Center, Ina-machi, Japan
[g] Department of Breast and Thyroid Surgery, Iwate Prefectural Central Hospital, Morioka, Japan
[h] Department of Breast Oncology, Miyagi Cancer Center Hospital, Natori, Japan
[i] Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan

ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 26 August 2014
Accepted 28 August 2014
Available online 29 August 2014

Keywords:
Androgen receptor
Aromatase inhibitor-resistance
Breast cancer
PSA
DDC

ABSTRACT

Aromatase inhibitors (AI) are commonly used to treat postmenopausal estrogen-receptor (ER)-positive breast carcinoma. However, resistance to AI is sometimes acquired, and the molecular mechanisms underlying such resistance are largely unclear. Recent studies suggest that AI treatment increases androgen activity during estrogen deprivation in breast carcinoma, but the role of the androgen receptor (AR) in breast carcinoma is still a matter of controversy. The purpose of this study is to examine the potential correlation between the AR- and AI-resistant breast carcinoma. To this end, we performed immunohistochemical analysis of 21 pairs of primary breast carcinoma and corresponding AI-resistant recurrent tissue samples and established two stable variant cell lines from ER-positive T-47D breast carcinoma cell line as AI-resistance models and used them in *in vitro* experiments. Immunohistochemical analysis demonstrated that the expression of prostate-specific antigen (PSA) and Ki-67 were significantly higher and ER and progesterone receptor (PR) were lower in recurrent lesions compared to the corresponding primary lesions. Variant cell lines overexpressed AR and PSA and exhibited neither growth response to estrogen nor expression of ER. Androgen markedly induced the proliferation of these cell lines. In addition, the expression profile of androgen-induced genes was markedly different between variant and parental cell lines as determined by microarray analysis.

These results suggest that in some cases of ER-positive breast carcinoma, tumor cells possibly change from ER-dependent to AR-dependent, rendering them resistant to AI. AR inhibitors may thus be effective in a selected group of patients.

1. Introduction

Breast cancer is one of the most common malignancies in women worldwide. The estrogen receptor (ER) is expressed in approximately two-thirds of breast carcinomas, and a great majority of ER-positive cases respond well to endocrine therapy. Aromatase inhibitors (AI), such as anastrozole, exemestane, and letrozole, potently block estrogen biosynthesis from androgens;

Abbreviations: AI, aromatase inhibitor; AR, androgen receptor; ARE, androgen-response element; DDC, L-DOPA decarboxylase; DHT, dihydrotestosterone; ER, estrogen receptor α; ERE, estrogen-response element; E_2, estradiol; GFP, green fluorescent protein; LI, labeling index; PR, progesterone receptor; PSA, prostate-specific antigen; TS, testosterone.

* Corresponding author at: Department of Molecular and Functional Dynamics, Center for Regulatory Epigenome and Diseases, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Tel.: +81 22 717 8680; fax: +81 22 717 7913.
E-mail address: shin@med.tohoku.ac.jp (S.-i. Hayashi).

http://dx.doi.org/10.1016/j.jsbmb.2014.08.019
0960-0760/

these agents have shown better clinical outcomes than anti-estrogen tamoxifen in several clinical trials [1–3]. Nevertheless, in some cases, acquired resistance has been reported after initial successful AI treatment [4]. The molecular mechanisms underlying AI resistance have been examined by several groups [5–9] but still remain largely unclear.

In contrast, androgens are considered to predominantly exert antiproliferative effects in breast carcinoma [10]. Recent meta-analysis showed that androgen receptor (AR) status was associated with better clinical outcomes in breast carcinoma patients [11]. However, the involvement of AR in promoting proliferation, especially in ER-negative cases [12,13], has been reported. Hickey et al. [14] have suggested that the AR rather than the ER plays an oncogenic role due to AR activation when estrogen activity is reduced.

Biologically active androgen, dihydrotestosterone (DHT), is produced locally in breast carcinoma. Aromatase acts as a negative regulator of *in situ* DHT production by decreasing its precursor [15]. Intratumoral DHT concentration was significantly higher in breast carcinoma treated with AI compared to untreated controls [16]. AI therapy is suggested to increase local androgen actions besides inducing estrogen deprivation [15,16]. Therefore, it is possible that intratumoral androgen activity may increase in recurrent breast carcinoma under estrogen deprivation caused by AI treatment, thus playing an important role in AI resistance. However, androgen activity has not been examined in recurrent breast carcinoma following AI therapy. In this study, we first examined the immunohistochemical features of recurrent breast carcinoma lesions during AI treatment and showed an increment of AR activity compared to the corresponding primary lesions. Moreover, we established AI-resistant model cell lines from ER-positive breast carcinoma cell line, T-47D, and further characterized biological roles of AR in the AI-resistant cell lines.

2. Materials and methods

2.1. Patients and tissues

ER-positive breast carcinoma specimens ($n = 21$) were obtained from postmenopausal women who underwent surgical treatment between 2002 and 2009 at: Tohoku University Hospital, Sendai, Japan ($n = 7$); Tohoku Kosai Hospital, Sendai, Japan ($n = 5$); Miyagi Cancer Center Hospital, Natori, Japan ($n = 5$); and Iwate Prefectural Central Hospital, Morioka, Japan ($n = 4$). The patient characteristics are summarized in Table 1. All patients received oral aromatase inhibitors after surgery and had asynchronous recurrence during this treatment. The median duration of treatment with AI was 34 months. 3 of the 17 patients who received anastrozol initially switched their treatment from anastrozol to exemestane because of an incidence of recurrence or side effect of anastrozol. The corresponding recurrent breast carcinoma lesions were available for examination in all cases. All specimens were fixed in 10% formalin and embedded in paraffin wax.

Our research protocol was approved by the Ethics Committee at Tohoku University School of Medicine and other institutional review boards.

2.2. Immunohistochemistry

Mouse monoclonal antibodies for AR (AR441) and Ki-67 (MIB1), and rabbit polyclonal antibody for prostate-specific antigen (PSA; IR514/IS514) were purchased from DAKO (Carpinteria, CA, USA). Amplification was performed using the Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin–biotin amplification method. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 76), and 0.006% H_2O_2) and counterstained with hematoxylin.

Immunohistochemistry to detect expression of the ER (CONFIRM anti-ER (SP1), Roche Diagnostics Japan, Tokyo, Japan) and progesterone receptor (PR; CONFIRM anti-PgR (1E2), Roche Diagnostics, Japan) was performed using the Ventana Benchmark XT instrument (Roche Diagnostics, Japan). Immunohistochemical analysis of HER2 expression was performed using HercepTest™ (DAKO).

Table 1
Clinicopathological characteristics of 21 patients in the present study.

	Number of patients	Median (min-max)
Patient age at the surgery[a]		63 (48-72)
Stage		
I	5	
II	9	
III	7	
Histological type		
Invasive ductal carcinoma	19	
Invasive lobular carcinoma	2	
Intrinsic subtype[b]		
Luminal A	16	
Lumbinal B	5	
Histological grade		
1	3	
2	15	
3	3	
Chemotherapy received		
Neoadjuvant Chemotherapy	3	
Adjuvant chemotherapy	7	
Not received	12	
Type of AI received after the surgery		
Anastrozole	17	
Exemestane	6	
Letrozole	1	
Time from the surgery to recurrence[a] (months)		53 (7-76)
Duration of AI-treatment[a] (months)		34 (7-70)
Recurrent lesions examined		
Lymph node	11	
Chest wall	5	
Lung	4	
Bone	1	

[a] Data represent the median (min–max), and all other values are presented as the number of cases.[b]Intrinsic subtype was defined according to 2011 St. Gallen surrogate definition [45].

2.3. Scoring of immunoreactivity

ER, PR, AR, and Ki-67 immunoreactivity was detected in the nuclei of breast carcinoma cells, and the percentage of immunoreactive cells, *i.e.*, labeling index (LI), was determined. PSA immunoreactivity was considered positive if any cytoplasmic staining was observed in the carcinoma cells [17]. HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest™, and specimens with a score of 3+ were considered positive. Moreover, *HER2* gene amplification was investigated by fluorescence *in situ* hybridization (FISH) in score 2+ cases.

2.4. Cells and reagents

T-47D breast carcinoma cells were stably transfected with the estrogen response element (ERE)-green fluorescent protein (GFP)

reporter plasmid as reported previously [18] (Supplementary Fig. S1A). T-47D cells were cultured in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 5% or 10% fetal calf serum (FCS; Tissue Culture Biogicals, Turale, CA, USA). Phenol red-free RPMI (PRF-RPMI; Gibco BRL, Grand Island, NY, USA) supplemented with 5% or 10% dextran-coated charcoal-treated FCS (DCC-FCS) was used as the steroid-depleted medium in each experiment. T-47D cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and authenticated using a PowerPlex® 16 STR system on January 25, 2013.

Estradiol, testosterone, DHT, bicalutamide, fulvestrant, and NSD-1015 (3-hydroxybenzylhydrazine dihydrochloride) were purchased from Sigma–Aldrich.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.019.

2.5. Real-time PCR

Total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan), and the extracted RNA was converted to cDNA using a

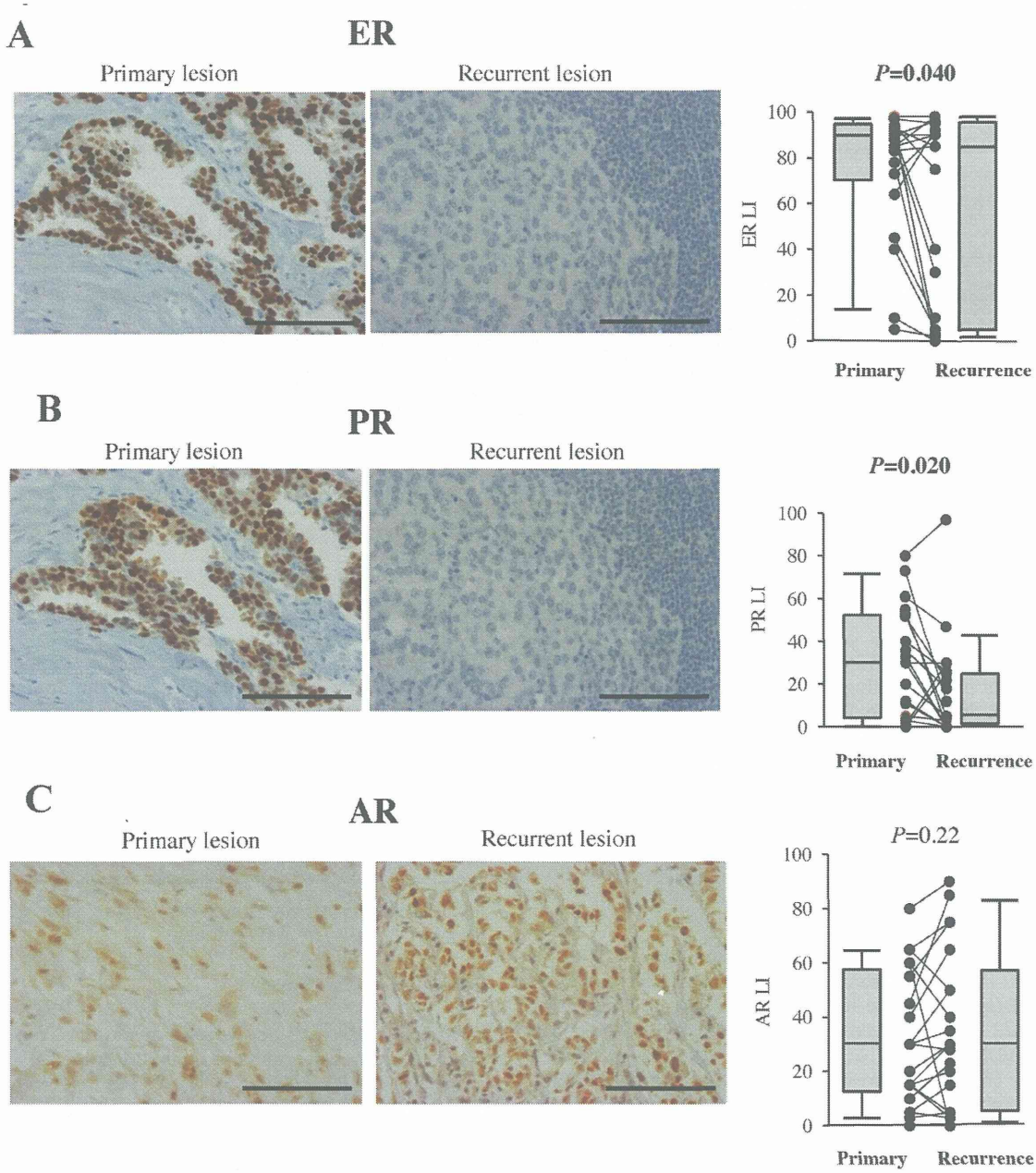

Fig. 1. Immunohistochemical analysis in AI-resistant breast carcinoma. Left panels show immunoreactivity of ER (A), PR (B), AR (C), PSA (D), and Ki-67 (E) in primary lesions, and middle panels show the same in corresponding recurrent lesions from the same patients during AI treatment. Left and middle panels of A and B show the same area. Bar = 100 μm. Right panels summarize changes in immunoreactivity in 21 paired breast carcinoma tissues obtained from primary and recurrent lesions. Each value is indicated by a slid circle, with lines connecting paired values from the same patient. The grouped data are represented as box-and-whisker plots. In A–C and E, the median value is shown by a horizontal line in the box plot, and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles, respectively. Statistical analyses were performed using a Wilcoxon signed-rank test, and $P < 0.05$ (bold) was considered significant.

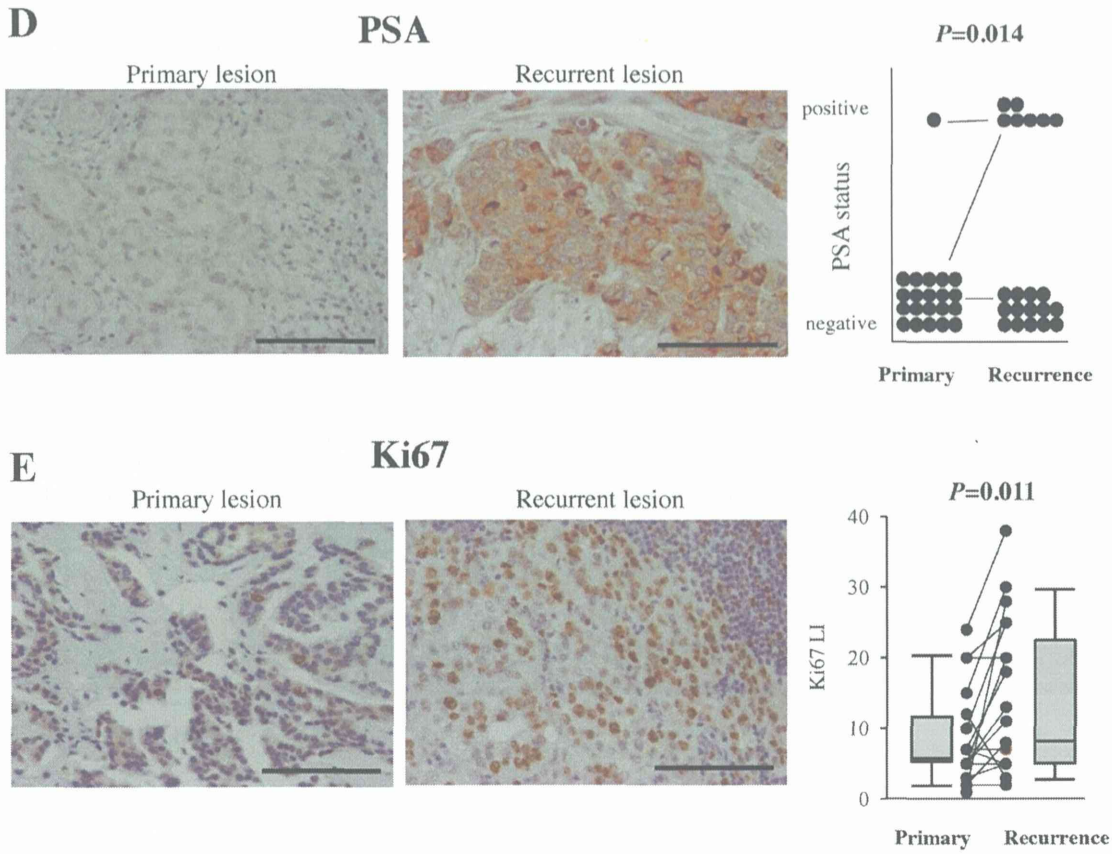

Fig. 1. (Continued)

QuantiTect reverse transcription kit (QIAGEN, Mississauga, Ontario, Canada). A 2-μl aliquot was used as a template for real-time PCR, which was performed according to the manufacturer's protocol using the Applied Biosystems Step One Real-time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). The expression of the target gene relative to the *RPL13A* internal control was calculated. The primer data are summarized in Supplementary Table S1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.019.

2.6. Immunoblotting

Proteins were extracted using Complete Lysis-M (Roche, Indianapolis, IN, USA). Protein extracts (10 μg) were subjected to SDS-PAGE (Super Sep Ace 10%, Wako Pure Chemical Industries, Osaka, Japan) and transferred onto a membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare, Buckinghamshire, UK). The primary antibodies were anti-AR (#3202), anti-PSA (#5365), and anti-β-tubulin (#2146) (Cell Signaling Technology, Tokyo, Japan) and anti- ER antibody (sc-7207; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit, was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Antibody–protein complexes were detected using Immun-Star™ AP substrate (Bio-Rad Laboratories), and the protein bands were visualized using the ImageQuant™ LAS 4000 image analyzer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.7. Luciferase reporter assay

The estrogen response element reporter plasmid, ERE-tk-Luci, was used as described previously [19]. The androgen response element (ARE) reporter plasmid, pGLPSAp5.8 [20] , containing the PSA–ARE was kindly provided by Dr. Mizokami (Kanazawa University, Kanazawa, Japan). The control vector pRL-TK (Promega, Madison, WI, USA) was used as an internal control for transfection efficiency. The luciferase assay was performed according to a previous report [19] with some modifications. Cells were cultured in a steroid-depleted medium for 3 days before the transfection using TransIT LT-1 reagent (Mirus, Madison, WI, USA), and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.8. Cell proliferation assay

After 3 days in steroid-depleted medium, cells were seeded in 24-well culture plates at a density of 20,000 cells/well with drugs and hormones for 4 days. Cells were then harvested and counted using a Sysmex CDA-500 automated cell counter (Sysmex, Kobe, Japan).

2.9. Microarray analysis

Whole Human Genome DNA Microarray 4 × 44 K ver. 2.0 (Agilent Technologies, Santa Clara, CA, USA) was used in this study. Cells were cultured in steroid-depleted medium for 3 days, followed by a treatment with 1 nM DHT with or without 10 μM